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Decay of the glycolytic pathway and adaptation to intranuclear parasitism within Enterocytozoonidae microsporidia

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1 **Originality-Significance Statement:** This study uses genomics to explore
2 metabolic reduction of microsporidia in the highly economically important
3 Enterocytozoonidae family, showing that uniquely amongst eukaryotes, this whole clade has
4 lost any clear intrinsic means of generating ATP. It also presents the first genome sequence of
5 an obligate intranuclear eukaryotic parasite and the first sequence for the shrimp pathogen
6 EHP, which is crucial to underpinning the development of new tools to diagnose, monitor and
7 potentially mitigate the negative impacts of this major emerging aquacultural pathogen.

8

9 **Summary:**

10 Glycolysis and oxidative phosphorylation are the fundamental pathways of ATP generation in
11 eukaryotes. Yet in microsporidia, endoparasitic fungi living at the limits of cellular
12 streamlining, oxidative phosphorylation has been lost: energy is obtained directly from the
13 host or, during the dispersive spore stage, via glycolysis. It was therefore surprising when the
14 first genome from the Enterocytozoonidae – a major family of human and animal-infecting
15 microsporidians - appeared to have lost glycolysis. Here we sequence and analyse genomes
16 from four additional members of this family, shedding new light on their unusual biology.
17 Our survey includes the genome of *Enterocytozoon hepatopenaei*, a major aquacultural
18 parasite currently causing substantial economic losses in shrimp farming, and *Enterospora*
19 *canceri*, a pathogen that lives exclusively inside the nuclei of its crab host. Our analysis of
20 gene content across the clade suggests that *Ent. canceri*'s adaptation to intranuclear life is
21 underpinned by the expansion of transporter families. We demonstrate that this entire lineage
22 of pathogens has lost glycolysis and, uniquely amongst eukaryotes, lacks any obvious
23 intrinsic means of generating energy. Our study provides an important resource for the
24 investigation of host-pathogen interactions and reductive evolution in one of the most
25 medically and economically important microsporidian lineages.

26

27 **Introduction:**

1 The microsporidia are important emergent pathogens of almost all known animal phyla in all
2 major biomes (Stentiford et al., 2016). The Enterocytozoonidae family is home to two of the
3 most economically important microsporidian species. *Enterocytozoon bieneusi* is the most
4 prevalent human infecting microsporidian with some studies reporting prevalence of 58% in
5 immunocompetent individuals. *Enterocytozoon hepatopenaei* is a recently emerged pathogen
6 of the farmed shrimp species *Penaeus monodon* and *Penaeus vannamei* and severely retards
7 the growth of the shrimp and has rapidly spread across south-east Asia (Newman, 2015).
8 Microsporidia are also pre-eminent model systems for understanding the processes of
9 metabolic, cellular and genomic reduction in eukaryotes, with genomes as small as 2.3 Mb
10 and as few as 1,990 encoded genes (Corradi et al., 2010). A large set of core eukaryotic genes
11 were jettisoned early in the evolutionary history of microsporidia (Nakjang et al., 2013). This
12 left them without a mitochondrial genome and without the ability to generate ATP via
13 oxidative phosphorylation (Williams et al., 2002; Nakjang et al., 2013), whilst their close
14 relative *Mitosporidium daphniae* retains a mitochondrial genome and components of the
15 electron transport chain (Haag et al., 2014). With the absence of oxidative phosphorylation in
16 microsporidia, glucose is only partially metabolised via glycolysis to release 7 % of its full
17 ATP potential (Berg et al., 2007). However, this energy supply is complemented by the
18 import of ATP molecules from the host with the help of horizontally acquired ATP/ATP
19 translocases (Richards et al., 2003; Tsaousis et al., 2008; Nakjang et al., 2013; Hacker et al.,
20 2014). Thus, microsporidians are considered to be entirely reliant on glycolysis and ATP
21 import from their hosts for energy (Heinz et al., 2012; Haag et al., 2014). One emerging idea
22 is that glycolysis occurs primarily in the spore stage but shuts down in intracellular life stages,
23 where the parasite can tap into the host's energy pools (Heinz et al., 2012). This is based on
24 data that demonstrate that the enzyme responsible for the first ATP-forming reaction in
25 glycolysis is enriched in the cytosol of the extracellular spores in comparison to the cytosol of
26 the intracellular meront stages of *Trachipleistophora hominis* (Heinz et al., 2012). Moreover,
27 the two enzymes responsible for the reoxidation of NADH for glycolysis (Alternative oxidase

1 and glycerol-3-phosphate dehydrogenase) were found to accumulate in the cytosol of
2 *Antonospora locustae* spores in a study by Dolgikh *et al.*, 2011.

3
4 When the genome of the human infecting microsporidian *E. bieneusi* was deeply surveyed
5 using whole-genome shotgun sequencing, there was a surprising revelation that most genes
6 involved in glycolysis were absent, in addition to the loss of those involved in the oxidative
7 phosphorylation pathway (Akiyoshi *et al.*, 2009; Keeling *et al.*, 2010). This implies that this
8 parasite relies entirely on ATP import from its host with no obvious means of generating
9 ATP, even in the extracellular spore stage (Akiyoshi *et al.*, 2009; Keeling *et al.*, 2010).

10
11 As organisms with highly reduced metabolisms, microsporidians are highly dependent on
12 their hosts and have an expanded repertoire of transport proteins that allow them to exploit the
13 rich environment in the host cell cytoplasm. Interestingly, meronts of *E. bieneusi* form tight
14 physical associations with the host mitochondria, and this, in other microsporidian species,
15 has been shown to maximise the host ATP-harvesting efficiency of the parasite (Desportes *et al.*,
16 1985; Hacker *et al.*, 2014). However, this association of microsporidian life stages with
17 host mitochondria is not universal across the group (Hacker *et al.*, 2014). In fact, some of the
18 aquatic host-infecting microsporidian species belonging to the Enterocytozoonidae family
19 inhabit the host nucleus rather than the cytoplasm. These species include *Enterospora*
20 *canceri*, *Enterospora nucleophila*, several members of the genus *Nucelospora*, and,
21 *Desmozoon lepeophtherii* (= *Paranucleospora theridion*) (Chilmonczyk *et al.*, 1991; Hedrick
22 *et al.*, 1991; Lom and Dykova, 2002; Stentiford and Bateman, 2007; Stentiford *et al.*, 2007;
23 Freeman and Sommerville, 2009; Nylund *et al.*, 2010; Freeman *et al.*, 2013). These species
24 have no access to the host mitochondria so cannot use this strategy to maximise ATP uptake.

25
26 Despite the economic and evolutionary importance of the Enterocytozoonidae, at present, *E.*
27 *bieneusi* is the only member of the group with a sequenced genome. This leaves several
28 intriguing unanswered questions about this group. Firstly, what drove these taxa to inhabit the

1 host nucleus and, what are the metabolic benefits and parasite adaptations to life in this
2 unusual environment? Secondly, when was glycolysis was lost in the microsporidia and does
3 the absence of this pathway in *E. bieneusi* represent an anomaly within the phylum?

4
5 To better understand this intriguing parasite family we sequenced, annotated and analysed the
6 genomes of several members of the Enterocytozoonidae. These include the intranuclear crab
7 parasite *Enterospora canceri*, the cytoplasmic crab parasites *Hepatospora eriocheir* and
8 *Hepatospora eriocheir canceri*, and, the cytoplasmic shrimp parasite *Enterocytozoon*
9 *hepatopenaei*. The latter is of particular interest given its recent emergence and significant
10 economic impact on the global shrimp farming industry (Rajendran et al., 2016; Stentiford et
11 al., 2016). This pathogen is reported to have reached epidemic levels in shrimp farms across
12 South East Asia, where the infection results in slow shrimp growth (Thitamadee et al., 2016).
13 For this reason, this pathogen is currently a major threat to sustainable shrimp farming in Asia
14 (Thitamadee et al., 2016). Thus, our data provide a resource for understanding the metabolic
15 strategy for intranuclear existence, as well as a foundation for investigating host-parasite
16 interactions (and potential intervention strategies) for economically important
17 microsporidians such as *E. hepatopenaei*. Additionally, our genome data reveals that the
18 genes that encode glycolysis show a pattern of parallel decay across the Enterocytozoonidae
19 clade: All of these species have incomplete glycolytic pathways, but different genes have
20 been lost in each species, implying a common loss of the selective pressure to retain
21 glycolysis in these diverse species of microsporidia.

22 23 **Results and Discussion:**

24 **Establishing the interrelationships of the Enterocytozoonidae using a multi protein**
25 **phylogeny:** In order to investigate the phylogenetic relationships between our studied species
26 within the Enterocytozoonidae, we generated a 21-protein concatenated phylogeny. This
27 demonstrated the close relationships of the crustacean parasites *Ent. canceri*, and *E.*

1 *hepatopenaei* to the human pathogen *E. bieneusi*, with *Hepatospora* forming an outgroup to
2 these (Figure 1 + 2 and Supplementary figure 1). This phylogeny implies that there has been a
3 rapid change in lifestyle within the group with drastic switches in both cellular localisation (*E.*
4 *hepatopenaei* and *E. bieneusi* are cytoplasmic, while *Ent. canceri*, is nuclear) and, host type
5 (*E. hepatopenaei* and *Ent. canceri* in aquatic crustaceans, and *E. bieneusi* in humans,
6 mammals and birds) over a relatively short evolutionary timescale. The finding that *E.*
7 *bieneusi* is phylogenetically nested amongst microsporidians with aquatic hosts suggests that
8 this species has emerged to infect human and terrestrial animal hosts via zoonotic transfer
9 from an aquatic environment.

10
11 **Parallel decay of the glycolysis and other metabolic pathways across the**
12 **Enterocytozoonidae:** The discovery that *E. bieneusi* lacked a functional glycolytic pathway
13 opened up a number of questions about how these organisms fuel their complex lifecycle
14 (Akiyoshi et al., 2009; Keeling et al., 2010). One of these was whether the loss was unique to
15 this species or had occurred more broadly within the Microsporidia. Firstly we took a
16 conserved protein set identified by Keeling et al. (Keeling et al., 2010). We used OrthoMCL
17 to cluster all publically available microsporidia predicted open reading frames and clusters
18 representing these conserved gene sets were used to populate supplementary table 1. In a
19 second approach, the same conserved protein set was used to interrogate our predicted ORFs
20 and our raw contigs for the Enterocytozoonidae using BLASTP and TBLASTN. This
21 revealed that that all surveyed representatives of the Enterocytozoonidae lacked multiple
22 components of the glycolytic pathway. We find 4 glycolytic enzymes retained in *Ent. canceri*,
23 2 in *E. hepatopenaei* and 4 in each of the *H. eriocheir* genomes. Although none of these
24 genomes were sequenced to completion, coverage of our genomes is high (Table 1) and it is
25 unlikely that our observations can be explained by a failure to sequence the missing
26 components of glycolysis. Using the presence or absence of a set of 44 broadly conserved
27 genes (transcriptional control proteins) to gauge genome completeness, we estimate that our
28 assemblies are 84-100% complete [*H. eriocheir canceri* (40/44), *H. eriocheir* (40/44), *Ent.*

1 *canceri* (42/44), *E. hepatopaenei* (43/44)] (Supplementary table 1). Assuming a genome
2 completeness of between 84% and 100%, we calculate the probabilities of observing at least
3 this many glycolytic losses simply as the result of incomplete sequencing (rather than true
4 absences) to be 2.869×10^{-08} for *Ent. canceri*, 6.975×10^{-10} for *E. hepatopaenei*, 8.629×10^{-05}
5 for *H. eriocheir canceri* and 8.629×10^{-05} for *H. eriocheir*. In addition, the genomes from our
6 two independently sequenced and assembled *Hepatospora eriocheir* sub-species share the
7 same pattern of glycolytic enzyme loss corroborating our assumptions that these gene
8 absences are true losses and not a consequence of incomplete sequencing. Overall, the data
9 suggest a common absence of a functional glycolytic pathway in the Enterocytozoonidae. Our
10 data also show that this loss did not occur as a single ancestral event but highlights a pattern
11 of parallel decay of the pathway with different genes lost in each lineage (Figure 2). This
12 suggests a relaxation of the selective pressure to keep glycolysis at the base of the
13 Enterocytozoonidae followed by pathway decay via loss of different enzymes. What is
14 common to each species is that one or more genes encoding a hexokinase-like protein are
15 retained, along with genes coding for a handful of other components. However and crucially,
16 phosphoglycerate kinase and pyruvate kinase, enzymes that catalyze the ATP-generating steps
17 of glycolysis, are consistently missing.

18
19 The conservation of ATP-expensive pyrimidine and purine synthesis pathways (Figure 3)
20 together with the absence of glycolytic enzymes involved in ATP-generating steps and the
21 retention of seemingly functional hexokinases (Figure 4) suggests that the cytosol of these
22 Enterocytozoonidae lineages represent a potential ATP sink. One consequence of this is an
23 increase in ADP/ATP ratio within the cytosol, which has been shown in *Chlamydiae* to
24 stimulate ATP uptake via ATP/ADP translocases (Trentmann et al., 2007). Thus, a high
25 ADP/ATP ratio in the microsporidian meront potentially generated by the broken glycolytic
26 pathway and ATP-intensive pathways such as pyrimidine and purine synthesis pathways may
27 be responsible for stimulating ATP import into the microsporidia via ATP/ADP translocases.

1 Other core metabolic enzymes were identified and mapped onto the multi-protein phylogeny.
2 Whilst individual microsporidian lineages have lost enzymes of different metabolic pathways
3 independently, the Enterocytozoonidae have undergone the most dramatic metabolic
4 reduction described to date. In addition to the loss of most glycolytic enzymes, they have also
5 lost the pentose phosphate pathway and most genes involved in fatty acid and trehalose
6 metabolism (Figures 1 & 2).

7
8 **The nature of microsporidian hexokinases in non-glycolytic lineages:** Within the
9 microsporidian phylum hexokinase is found either in single or multiple copies. Based on
10 sequence similarity, we identified hexokinase-like genes in the Enterocytozoonidae that were
11 divergent in comparison to other microsporidian hexokinases. This may reflect the fact that
12 they may not be functioning in the same pathway as those present in glycolytic microsporidia.
13 As seen previously in other microsporidia (Nakjang et al., 2013), our data shows a pattern of
14 independent gene duplications of these hexokinases across the Enterocytozoonidae, including
15 multiple genes in *E. bieneusi*, single gene copies in *Ent. canceri* and *E. hepatopenaei* and a
16 duplication event at the base of the *Hepatospora* lineage (Figure 4). An attractive explanation
17 for the retention and expansion of the hexokinase gene family in these seemingly non-
18 glycolytic organisms is that the redundant copy is secreted to modify host metabolism and
19 increase metabolic rewards for the pathogen as has been previously hypothesized to occur in
20 other glycolytic microsporidia (Cuomo et al., 2012). However, no signal sequences that
21 would typically direct these proteins to the secretory pathway were detected in any of the
22 Enterocytozoonidae hexokinase proteins using either SignalP or WOLFPSORT (Horton et al.,
23 2007; Petersen et al., 2011).

24
25 Interestingly, a protein tyrosine phosphatase A (PTPA) domain was found to be fused to the
26 N-terminus of the single copy of the *Ent. canceri* divergent hexokinase, the significance of
27 which is unclear. In other organisms, these tyrosine phosphatases perform regulatory roles by

dephosphorylating specific tyrosine residues (Tonks, 2006). One possibility is that the *Ent. cankeri* hexokinase has acquired a similar regulatory role.

The divergent nature of the gene sequences of these hexokinases suggested that they might no longer be functioning as typical hexokinases. To investigate this, we compared amino acid sequence conservation across known active sites between the new members of the Enterocytozoonidae sequenced in this study, hexokinases from published microsporidian genomes and the hexokinase 1 (HXK1) of *Saccharomyces cerevisiae*. In cases where a genome of a species encodes multiple hexokinase homologues, the additional hexokinase copies had lost some of the active sites identified in *S. cerevisiae* HXK1. Similarly, amino acid residues known to be important for the function of the *S. cerevisiae* HXK1 (Kuser et al., 2008) were frequently substituted or even deleted (Figure 4). As such, it is possible that, for these genomes, duplicate hexokinases may be non-functional. Taken together, these observations suggest that at least one hexokinase copy in each microsporidian genome, including members of the Enterocytozoonidae retained its active sites indicating that they may be capable of performing a conserved hexokinase function.

Inferring the role of the conserved hexokinase gene in the metabolically reduced genomes of the Enterocytozoonidae: Hexokinase catalyzes phosphorylation of glucose to glucose-6-phosphate (G6P). G6P is not recognized by glucose transporters , but in model organisms is channeled into one of at least four metabolic pathways: glycolysis, dolichyl-P-mannose synthesis, trehalose synthesis and the pentose phosphate pathway (Figure 3) (Mueckler, 1994).

Our gene content analysis demonstrates that out of these four metabolic pathways, only the dolichyl-P-mannose (DPM) synthesis pathway has a complete repertoire of enzymes (See figure 3) across representatives of the Enterocytozoonidae (See figure 3). The retention of the

1 dolichyl-P-mannose synthesis pathway in these metabolically reduced lineages could be due
2 its important role in the production of building blocks for biosynthesis and protein
3 glycosylation (Wujek et al., 2004; Shental-Bechor and Levy, 2008; Vagin et al., 2009;
4 Moharir et al., 2013).

5
6 In the pentose phosphate pathway, only glucose-6-phosphate dehydrogenase (G6PD) and
7 hexokinase were retained across all members of the Enterocytozoonidae, suggesting an
8 alternative role for the coupled reaction catalyzed by these two enzymes. In this context, it is
9 interesting to note that the reducing equivalent, NADPH, released during the conversion of
10 glucose-6-phosphate to 6-phosphogluconolate by G6PD is key for lipid synthesis and
11 scavenging harmful reactive oxygen species (ROS). As intracellular parasites, microsporidia
12 are subject to challenge by host defense mechanisms including ROS (Leiro et al., 2001). We
13 hypothesize that hexokinase and G6PD may be retained, in part, due to their role in protecting
14 the parasite against damage from host-derived ROS. Retention of the ROS pathway in all
15 analyzed species in this study is consistent with this hypothesis (Figure 3).

16
17
18 **Absence of an intrinsic ATP generating mechanism in the Enterocytozoonidae:** The
19 extreme metabolic reduction observed in these *Enterocytozoonidae* lineages suggest they are
20 completely reliant on horizontally acquired ATP/ADP translocases that are universal in the
21 Microsporidia for ATP acquisition from the host cell (Haag et al., 2014). These would
22 provide ATP for the Enterocytozoonidae merogonial stages, but it does not explain how these
23 lineages acquire or generate ATP during their extracellular spore stage for the presumably
24 energetic process of spore germination (Vavra and Lukes, 2013).

25
26 To explore the possibility that microsporidia have retained alternative ATP generation
27 pathways, we used BLAST searches to look for enzymes involved in alternative exergonic
28 metabolic pathways such as mixed acid fermentation, the Entner-Doudoroff pathway, and

lipid and protein oxidation. However, none of the microsporidian genomes surveyed had the hallmark enzymes of these pathways either. In addition, each of these pathways feeds into the citric acid cycle, which is absent in the Microsporidia.

Phagocytosis has been observed as an alternative form of host cell invasion by some microsporidia (Franzen et al., 2005). There is also evidence to suggest that host cell types parasitized by the presently studied Enterocytozoonidae lineages (hepatopancreatic epithelial cells and mammalian enterocytes), have phagocytic properties (Alday-Sanz et al., 2002; Neal et al., 2006; Bu et al., 2010; Liu et al., 2013). It is therefore possible that the spores of the Enterocytozoonidae lineages could invade their respective host cells by phagocytosis. This would bring the microsporidian into contact with the host ATP supply and if ATP/ADP translocases were expressed at this time, they could provide the spore with the ATP needed to germinate and to escape from the phagocytic vesicle. As mentioned, above, a truncated glycolytic pathway could potentially stimulate ATP uptake in the microsporidian by generating a high ADP/ATP ratio. In the case of *Ent. canceri*, entry into the host cell by phagocytosis would need to be followed by a second step in which the parasite gains access to the nucleoplasm, where it completes its lifecycle. One possibility is that *Ent. canceri* could enter the nucleus by means of polar tube extrusion following germination in the cytoplasm. It is worth noting that members of the Cryptomycota (Syn. Rozellomycota), a related group of parasitic fungi, are able to gain access to the nucleoplasm without a polar tube (Michel et al., 2000; Michel et al., 2009; Corsaro et al., 2014) although the mechanism by which they do so remains unknown. Phagocytosis is typically mediated by pathogen ligand binding to phagocytic receptor. If this is the main means of host cell invasion by these Enterocytozoonidae lineages, then identifying and blocking the appropriate ligands may inhibit pathogen entry into the host cell and provide a means of controlling infection.

Comparing the plasma membrane transporter repertoire of nuclear and cytoplasm-infecting microsporidians: To investigate the adaptation of *Ent. canceri* to its unique

intranuclear niche, we compared the predicted complement of membrane transporters across the Enterocytozoonidae to determine whether the intranuclear *Ent. canceri* genome encoded a different transporter repertoire to that found in sister genera inhabiting the cytoplasm. Interestingly, the Enterocytozoonidae as a whole encode fewer homologues of characterised membrane transport proteins than other sequenced microsporidia (median 23, compared to 46 and 48 in the outgroups *Encephalitozoon cuniculi* and *Trachipleistophora hominis*, respectively), suggesting a reduction in transport repertoire in their common ancestor (Supplementary table 2). However, the branch leading to *Ent. canceri* experienced a substantial gain in characterised membrane transporters, encoding a number (45 transporters) similar to that of other microsporidians. These lineage-specific gains included additional representatives of the ABC, lipid (P-type ATPase), choline (CTL), amino acid, nucleotide sugar (UAA) and cation transporter families, raising the possibility that, against a background of extreme genome reduction, this increased transporter repertoire is associated with adaptation to the intranuclear environment.

The predicted secretomes of the Enterocytozoonidae: A key question related to members of the Enterocytozoonidae is how these closely-related organisms, with vastly different parasitic habits and host preferences, interact with their hosts. To screen for proteins secreted by these parasites into their host cells, we screened each of the predicted proteomes with SignalP 4.0 and identified those proteins with appropriate signal sequences to allow for translocation to the host cell cytoplasm (or nucleoplasm) (Petersen et al., 2011). We filtered out those proteins with multiple transmembrane domains that may be anchored into parasite membranes. Although this list is likely to include some proteins more generally associated with the secretory pathway or cell wall biosynthesis, we also expected to find potential effectors. Whilst dominated by hypothetical proteins, the list also included proteins with conserved domains allowing prediction of their function (Supplementary table 3). Predicted secreted proteins present in more than one Enterocytozoonidae genus, and thus representing candidate effectors include putative peptidyl-prolyl cis-trans isomerases and

glycosyltransferases, both protein families that have been identified as effectors in other microbes (Unal and Steinert, 2014; Lu et al., 2015).

This study has provided a major new resource for the investigation of host-pathogen interactions, phylogeny, genetic reduction and pathogen evolution in arguably the most economically and evolutionarily important families within the microsporidian phylum (Stentiford et al., 2016). We provide the first genome sequence for the major yield-limiting shrimp pathogen *E. hepatopenaei*. This genomic data in conjunction with previously-published information on the parasite's transmission cycle (Tangprasittipap et al., 2012; Tangprasittipap et al., 2013), a growing body of genomic data for host shrimp species (Baranski et al., 2014, You et al., 2010; Castillo-Juarez et al., 2015; Yu et al., 2015) and the capacity to study this pathogen in the shrimp host (Salachan et al., 2017) will undoubtedly underpin the development of new tools to diagnose, monitor and potentially mitigate the negative impacts of *E. hepatopenaei*. As a whole our data provide resounding evidence of the consistent loss of glycolysis into the Enterocytozoonidae. We demonstrate that glycolytic enzymes were not lost in a single event in the ancestor of the group but rather there has been a common loss of the selective pressure to retain glycolysis followed by a parallel erosion of the pathway by differential loss of the enzymes across the members of the genus. Microsporidia are already primary models of metabolic reduction in eukaryotes and the Enterocytozoonidae take this reduction even further making them the only eukaryotic group to have eliminated all canonical ATP-generating pathways. Whilst there are precedents for loss of glycolysis amongst prokaryotes that form very close host associations, for example *Mycoplasma hominis* and *Nanoarchaeum equitans*, (Waters et al., 2003; Pereyre et al., 2009), these organisms do not have an extracellular spore stage. Loss of glycolysis makes the Enterocytozoonidae unique amongst eukaryotes and leaves the enigma of how they complete their life cycle, surviving out of association with the host and activating their spores to invade host cells in the absence of their own energy generation system.

1

2 **Materials and Methods**

3 **Purification of *Enterospora canceri* and *Hepatospora eriocheir canceri* spores:** European
4 edible crab adults (*Cancer pagurus*) were purchased from local fishermen in Weymouth, UK
5 (50°34'N, 2°22'W) in January 2013. The hepatopancreases isolated from crabs infected with
6 either *Ent. canceri* or *H. eriocheir canceri* were crushed with a sterile pestle and mortar in 1 x
7 PBS. The homogenous mash was then filtered through a 100 µm mesh followed by cell
8 sieving through 40 µm filter and the filtrate was further purified using a Percoll density
9 gradient.

10 **Purification of *Enterocytozoon hepatopenaei* spores:** *E. hepatopenaei* infected shrimp
11 *Penaeus vannamei* were collected from commercial shrimp ponds in Thailand in January
12 2015. The size of the shrimp varied from 10-15 grams. The hepatopancreases were dissected
13 out, homogenised with a sterile ground-glass pestle, and filtered through 100 µm and 40 µm
14 cell strainers respectively to remove tissue debris. The filtered suspension was further purified
15 by a Percoll density gradient.

16 **Purification of *Hepatospora eriocheir* spores:** The hepatopancreas of an infected Chinese
17 mitten crab was passed through a 200 µm mesh and the filtrate was then again passed through
18 a 40 µm mesh. The filtrate was incubated in a 1 x PBS -0.05% Triton X-100 solution for 1 hr.
19 The washed and pelleted filtrate was then further purified using a Percoll density gradient.

20 **Genomic DNA extraction for sequencing:** For *H. eriocheir canceri*, *E. hepatopenaei* and
21 *Ent. canceri*, aliquots of purified spores were ground for 10 minutes in liquid nitrogen. This
22 step was repeated three times before resuspending the resulting powder in 800 µl of phenol
23 before proceeding with a standard phenol chloroform extraction followed by an ethanol
24 precipitation as per Campbell et al., 2013. For *H. eriocheir*, purified spores were subjected to
25 bead beating followed by phenol/chloroform extraction and ethanol precipitation as per
26 Campbell et al., 2013.

1 **Sequencing Protocols:** One *E. hepatopenaei* DNA sample was processed with NextFlex
2 Rapid protocol without PCR amplification (BIOO Scientific) and was sequenced using the
3 MiSeq v3 reagents 300 (PE). A second *E. hepatopenaei* DNA sample was processed for
4 PacBio Sequencing which was performed using PacBio RS II technology of a 10 kb library
5 on a single SMRT cell by Macrogen, South Korea. For *H. eriocheir* DNA a SPRIworks
6 fragment library (Beckman Coulter) was generated and this was sequenced on the HiSeq 2000
7 100 PE. *Ent. canceri* DNA was used to generate a SPRIworks fragment library (Beckman
8 Coulter), the first of these was sequenced using MiSeq v2 reagents 250 (PE) and the second
9 using MiSeq v3 reagents 300 (PE). *H. eriocheir canceri* DNA was used to generate a
10 SPRIworks fragment library (Beckman Coulter) which was sequenced using the MiSeq v2
11 reagents 250 (PE).

12 **Genome assembly and genome annotation:** *MiSeq and HiSeq data:* FASTQC was used to
13 identify and filter/trim reads with poor quality scores. A total of 197,235,682 reads with a
14 maximum length of 300 bp were used to assemble the *E. hepatopenaei* genome with the
15 Spades assembly pipeline (v.3) (Bankevich et al. 2012). A total of 222,450,433 paired-end
16 reads with a maximum length of 99 bp were used to assemble the genome of *H. eriocheir*
17 with the Velvet package (v1.1) (Zerbino & Birney 2008). A total of 8,740,870 paired-end
18 Illumina reads with a maximum length of 251 bp were used to assemble the *H. eriocheir*
19 *canceri* genome with the A5_miseq assembly pipeline (Coil et al. 2015). A total of 4,632,816
20 reads with a maximum length of 301 were used to assemble the *Ent. canceri* genome with the
21 A5_miseq assembly pipeline (Coil et al. 2015). All reads used had a mean quality score above
22 32. Protein prediction for sequenced genomes was performed with the MAKER annotation
23 package (Cantarel et al., 2008). To enable the program to make the best possible predictions
24 for the newly assembled genomes of *Ent. canceri*, *E. hepatopenaei*, *H. eriocheir* and *H.*
25 *eriocheir canceri*, the ab-initio protein prediction program incorporated within MAKER was
26 trained on the published genome of *E. bieneusi* as per user guide.

27 **PacBio data:** Subreads for the genome of *E. hepatopenaei* were assembled *de novo* by Canu
28 (version 1.3) (Berlin et al., 2015) with the default parameter setting

(<http://canu.readthedocs.io/>). Open-reading frames (ORFs), tRNAs and rRNAs were predicted using Prokka (version 1.11) (Seemann, 2014). Contaminating bacterial sequences found in the initial assembled reads were removed using a combination of both BLASTP and BLASTX searches for putative ORFs and by BLASTN searches for rRNAs against the NCBI nr and nt databases.

Identification of core metabolic genes: In order to assess how the protein repertoire encoded by the genomes of *Ent. canceri*, *E. hepatopenaei* and *Hepatospora* spp. sequenced in this study compared to that of other microsporidians, open reading frames (ORFs) of all four sequenced genomes and those from 19 publicly available microsporidian species were grouped into orthologous families with the command line program, OrthoMCL (Li et al., 2003) set on the following parameters: MCL inflation=1.1 and Maximum weight =100. These protein clusters were subsequently parsed for 381 core microsporidian proteins to assess completeness of newly sequenced genomes. This protein set has been previously used for a similar study by (Keeling et al., 2010). As a further verification for the presence/absence of this conserved gene set, BLASTP and TBLASTN searches were carried out on our newly predicted ORFS and newly generated genomic contigs. This revealed further predicted proteins that were not picked up by our OrthoMCL strategy or that were not predicted by our MAKER annotation.

Identification of transporter proteins from five genomes

ORFs from all four genomes sequenced in this study and from 19 publicly available microsporidian species were screened using the transmembrane domain prediction tool, TMHMM (Krogh et al., 2001) to select proteins with one or more transmembrane domains. Since the TMHMM program is known to erroneously identify signal peptides as transmembrane domains the protein set was also analysed with SIGNALP (Petersen et al., 2011). Proteins predicted to have a single transmembrane domain that was also identified to be a signal peptide were removed from the analysis. A BLASTP search with the remaining

protein set was carried out against the following databases: SGD, TCDB and NCBI databases (Saier et al., 2006; Cherry et al., 2012; Tatusova et al., 2014). Where possible, a consensus BLAST hit ID was selected for each putative transmembrane protein. WOLFPSORT (Horton et al., 2007) was subsequently used to predict a subcellular localization for these proteins. Predicted plasma membrane proteins that had four or more transmembrane domains were selected for further analysis as potential transport proteins.

For each predicted plasma membrane transporter, its OrthoMCL orthologous cluster was manually parsed to recover orthologs that may have been missed by the automated pipeline described above. The majority of the final predicted plasma membrane transporters (227/235) assigned to a functional group had four or more transmembrane domains. For predicted plasma membrane transporters that were not assigned to any functional group, only those with four or more transmembrane domains were retained. Although 18 non-Enterocytozoonidae microsporidian species were used in this analysis, only data for *Enc. cuniculi* and *T. hominis* were displayed in the final results.

Identification of secreted proteins/potential effectors

ORFs from the newly sequenced genomes and those from 19 other microsporidian species were parsed with the TMHMM command line tool (Krogh et al., 2001) to detect proteins with transmembrane domains. Proteins that were devoid of transmembrane domains or only possessed a single transmembrane domain were retained for further analyses. This protein set was passed to the signal peptide prediction program, SIGNALP (Petersen et al., 2011). Signal peptide-containing proteins were retained as potential secreted effectors. Finally, a Gene Ontology assessment of the predicted secreted proteins was performed with the graphical interphase version of BLAST2GO 3.2 (Conesa et al., 2005).

Phylogenetic analyses: Homologs for 21 universally conserved proteins: Wrs1p, Taf10p, TFIIE, Taf10p, Tfa2p, Sec62p, Abd1p, SPT16, Brn1p, Nob1p, Caf40p, Tfb2p, Bos1p, Npl4p, Tma46p, Tfa1p, Clp1p, Spt5p, Sec63p, Pri2p and Enp2p were aligned with MUSCLE

(v3.8.31) (Edgar, 2004) using the default settings and subsequently masked with the automatic command line tool TrimAl (v1.2rev59) (Capella-Gutierrez et al., 2009). The substitution model prediction option on MEGA (v6.06) (Tamura et al., 2011) was used to identify the best substitution models for the individual protein homolog sets. These models were then used to perform maximum likelihood analyses on the individual homolog protein sets using the command line program, RaxML (v7.2.7) (Stamatakis, 2014). These were pilot trees to check for unusually long-branch lengths indicative of unlikely orthologs. The individual masked alignments of the homolog protein sets were manually concatenated using Seaview (v4.5.4) (Gouy et al., 2010). A GTR+GAMMA module on RaxML (Stamatakis, 2006) was subsequently used to construct the final multi-protein concatenated phylogenetic tree.

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The authors have no conflicts of interest to declare

Availability of data

1 The Whole Genome Shotgun projects described in this paper have been deposited at
2 DDBJ/ENA/GenBank under the accessions LVKB000000000, LTAI000000000,
3 LWDP000000000 and MNPJ000000000. The versions described in this paper are
4 LVKB010000000, LTAI010000000, LWDP010000000 and MNPJ010000000.

5

6 Tables:

7 **Table 1: Genome statistics for the newly sequenced genomes.** *Gene set taken from
8 Desjardins et al., 2015

Statistics	<i>Hepatospora eriocheir</i>	<i>Hepatospora eriocheir canceri</i>	<i>Enterospora canceri</i>	<i>Enterocytozoon hepatopenaei</i>
Assembly size (Mb)	4.57	4.84	3.10	3.26
Contigs	1300	2344	537	64
Mean coverage (X)	4477.89	63.18	288	363
Contig N50 (bp)	17583	3349	11128	125008
GC content (%)	22.44	23.16	40.15	25.45
GC content coding (%)	25.58	25.41	41.95	27.81
Coding regions (%)	42.39	40.31	59.50	71.87
Splicing machinery *	7/29 genes	7/29 genes	7/29 genes	7/29 genes
Genes	2716	3058	2179	2540

9

10 Figure legends:

11 **Figure 1: Metabolic profiling of microsporidian genomes.** Filled squares represent
12 presence of a gene involved in a metabolic pathway whereas a blank space represents absence
13 of a gene. The phylogenetic positions in the cladogram are derived from maximum likelihood
14 analyses performed on a concatenated alignment of 21 conserved proteins. Values represent
15 levels of bootstrap support (100 replicates) for the corresponding nodes.

1

2 **Figure 2: Independent loss of glycolytic enzymes and retention of the hexokinase gene**
3 **across the Enterocytozoonidae family.** Filled circles represent the presence of a gene
4 whereas empty circles represent the absence of a gene. The loss of different glycolytic genes
5 in different lineages suggests that the selective pressure for the retention of glycolysis was
6 lost at the base of clade, leading to evolutionary decay of the pathway over time.

7

8 **Figure 3: Comparing the enzyme repertoire of key metabolic pathways between**
9 **members of the Enterocytozoonidae family and other microsporidians** (Figure adapted
10 from Nakjang et al., 2013. Thick black arrows represent those pathways found in all
11 Enterocytozoonidae. Where there is differential loss of the pathway across the
12 Enterocytozoonidae, presence of a gene in each species is represented by a different coloured
13 arrow. Predicted number of plasma membrane transporter families and their respective
14 substrates are also colour coded for each species of the Enterocytozoonidae.

15

16

17 **Figure 4: Phylogeny of hexokinase reveals that it is duplicated in some microsporidian**
18 **lineages.** The phylogenetic positions are derived from maximum likelihood analyses
19 performed on a masked alignment of hexokinase proteins. Mammalian hexokinases were used
20 to root the tree. Bootstrap support values that are above 95% (100 replicates) are represented
21 by black dots on the respective nodes. Similarity between *S. cerevisiae*'s hexokinase active
22 sites and those of other lineages are represented with a heat map.

23

24

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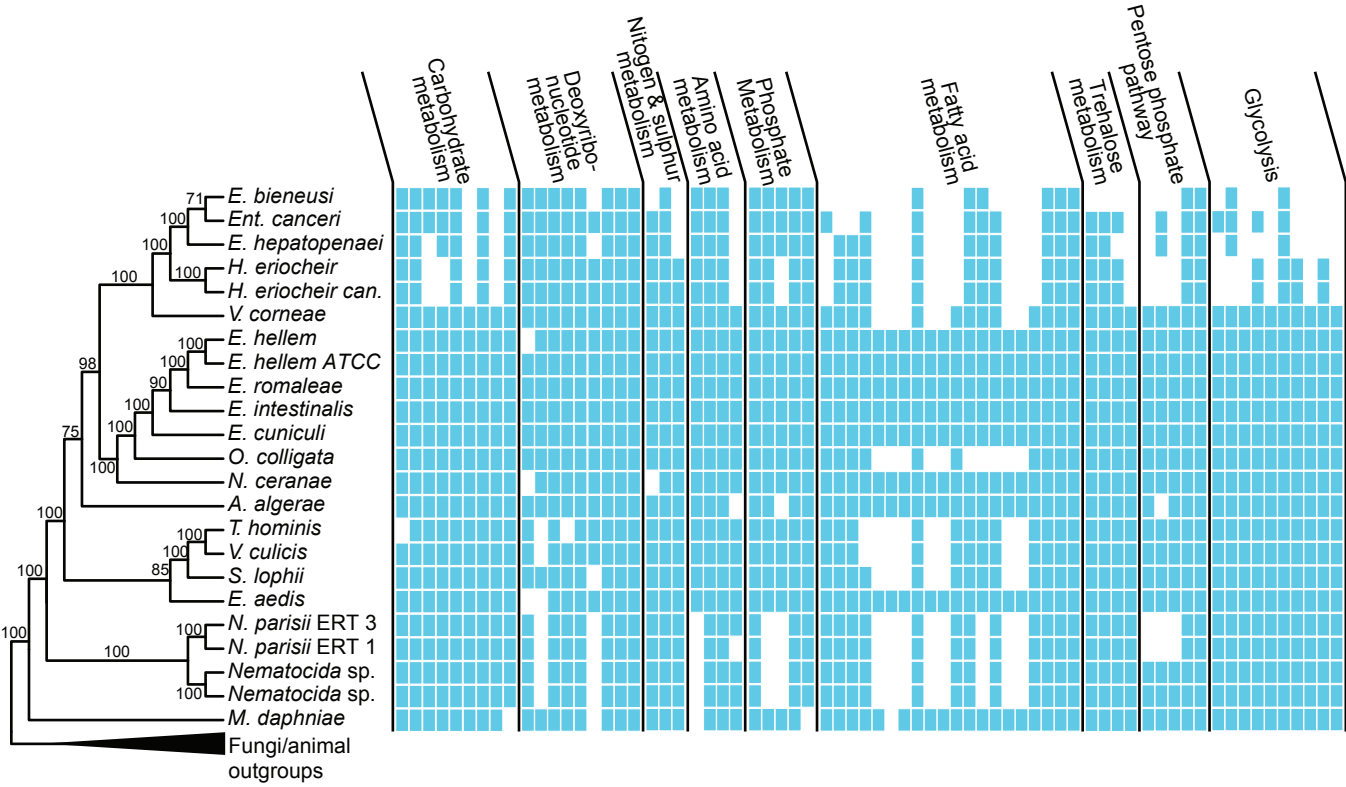
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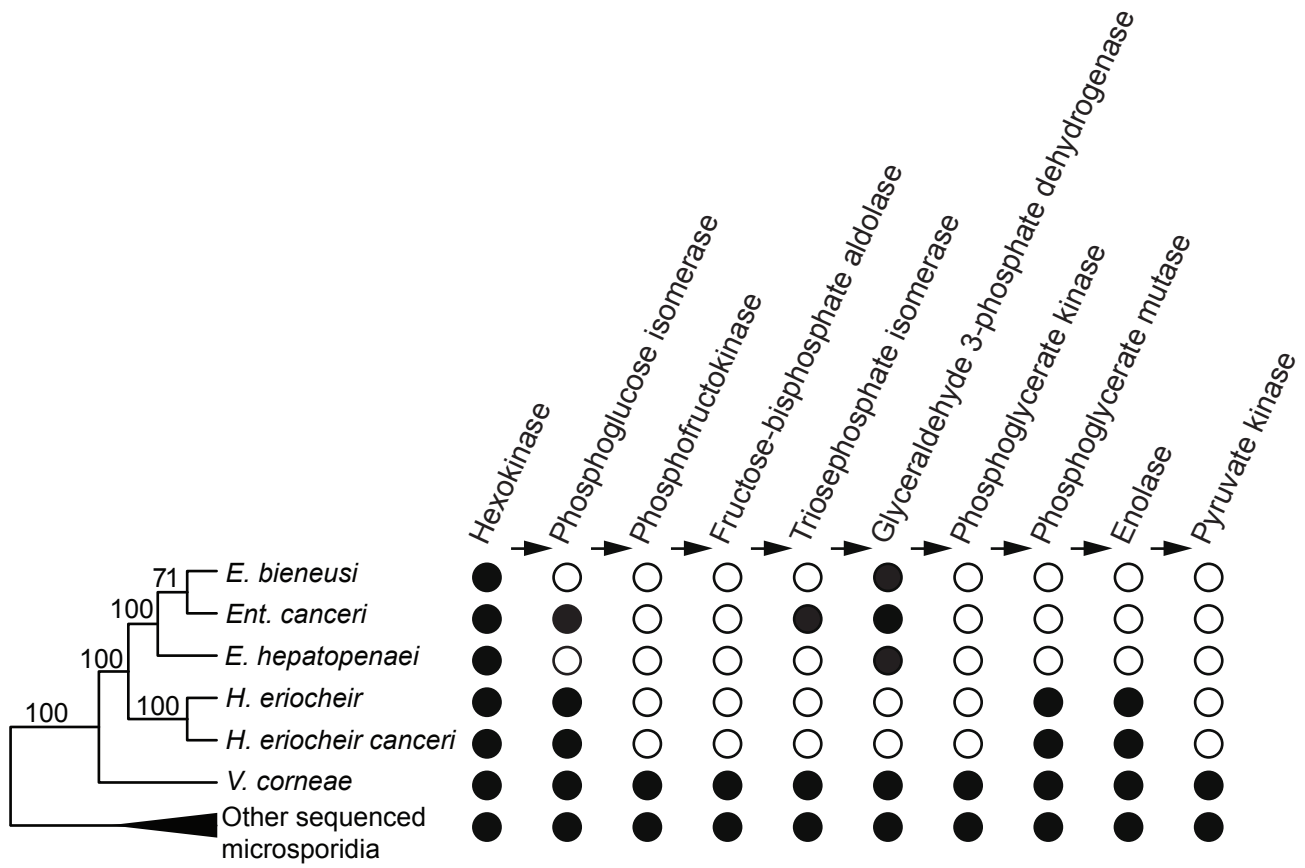
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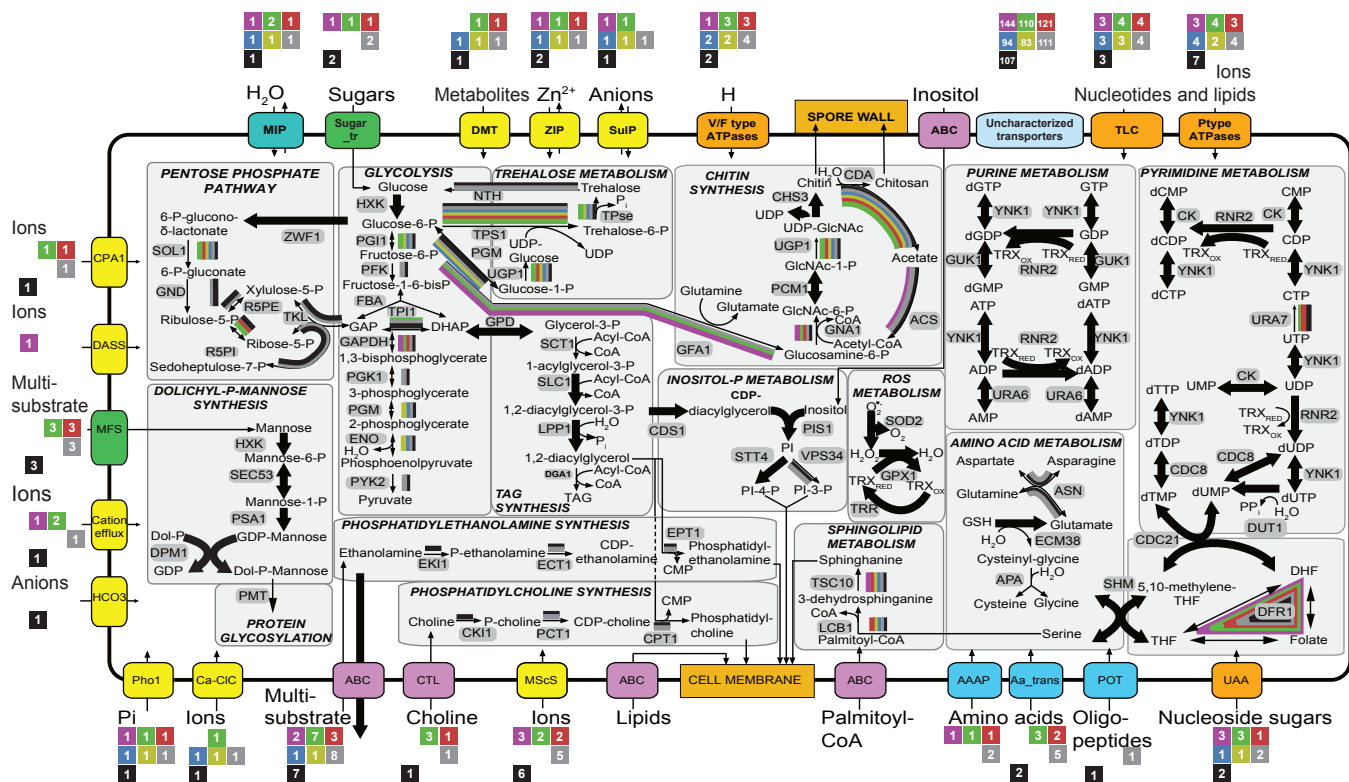
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4







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■ *E. hepatopenaei*
■ *H. eriocheir*
■ *H. eriocheir canceri*
■ *Enc. cuniculi*
■ *T. hominis*

Dol - Dolichyl
 TAG - Triacylglycerol
 GAP - Glyceraldehyde-3-phosphate
 GlcNAc - N-acetylglucosamine
 PI - phosphatidylinositol
 GSH - Glutathione
 THF - Tetrahydrofolate
 DHF - Dihydrofolate
 TRX - Thioredoxin

Cation efflux - Cation Efflux Pump
 HCO₃ - Anion Transport Protein
 MIP - Major Intrinsic Protein
 MFS - Major Facilitator Superfamily
 DMT - Drug/Metabolite Transporter
 ZIP - Zinc/Iron Permease
 SulP - Sulfate Permease
 AAAP - Amino Acid/Auxin Permease
 V/F type ATPases - Vacuolar Vesicular Proton Pump
 P type ATPases - E1-E2 ATPases
 Sugar_tr - Glucose transporter/Sugar transporter family
 Cation efflux - Cation Efflux Pump
 Ca CIC - Calcium Dependent Chloride Channels
 MscS - Small Conductance Mechanosensitive Ion Channel
 TLC - ATP/ADP Translocase
 UAA - UDP-N-acetylglucosamine Transporter
 ABC - ATP-Binding Casette
 CTL - Choline Transporter
 POT - Proton-Dependent Oligopeptide Transporter
 Pho1 - Phosphate transporter

